

Myocardin is a bifunctional switch for smooth versus skeletal muscle differentiation

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Skeletal and smooth muscle can mutually transdifferentiate, but little molecular insight exists as to how each muscle program may be subverted to the other. The myogenic basic helix–loop–helix transcription factors MyoD and myogenin (Myog) direct the development of skeletal muscle and are thought to be dominant over the program of smooth muscle cell (SMC) differentiation. Myocardin (Myocd) is a serum response factor (SRF) coactivator that promotes SMC differentiation through transcriptional stimulation of SRF-dependent smooth muscle genes. Here we show by lineage-tracing studies that Myocd is expressed transiently in skeletal muscle progenitor cells of the somite, and a majority of skeletal muscle is derived from Myocd-expressing cell lineages. However, rather than activating skeletal muscle-specific gene expression, Myocd functions as a transcriptional repressor of Myog, inhibiting skeletal muscle differentiation while activating SMC-specific genes. This repressor function of Myocd is complex, involving histone deacetylase 5 silencing of the Myog promoter and Myocd's physical contact with MyoD, which undermines MyoD DNA binding and transcriptional synergy with MEF2. These results reveal a previously unrecognized role for Myocd in repressing the skeletal muscle differentiation program and suggest that this transcriptional coregulator acts as a bifunctional molecular switch for the smooth versus skeletal muscle phenotypes.

deacetylase | myogenic | SRF | MEF2 | promoter

Skeletal muscle identity is controlled primarily by four skeletal muscle-specific myogenic regulatory factors (MRFs), MyoD, myogenin (Myog), Myf5, and MRF4, which cooperate with the myocyte enhancer factor-2 (MEF2) transcription factor to activate skeletal muscle gene expression (1). Although the MRFs act in a dominant manner and can convert a variety of cell types, including smooth muscle, into skeletal muscle (2), there are settings in which skeletal muscle can be induced to transdifferentiate into other cell types, suggesting that the MRFs may be subordinate to other cell-specific transcription factors (3, 4).

Much of the work related to transcriptional regulation of smooth muscle cell (SMC) differentiation has focused on serum response factor (SRF), a widely expressed transcription factor that binds the CArG box found in the regulatory regions of many SMC-specific genes (5). Genetic inactivation of SRF (6) and CArG mutagenesis studies in transgenic mice (7) have confirmed the necessity of CArG-SRF in controlling SMC differentiation. However, SRF is only a weak transcriptional activator and requires interacting cofactors that recruit proteins to promote a permissive state for gene transcription. One such cofactor is myocardin (Myocd), which is expressed primarily in cardiac and SMCs and displays high transcriptional activity (8). Myocd can activate SMC-specific genes (9), and genetic deletion of Myocd in mice leads to defective vascular SMC differentiation (10). Thus, Myocd displays features of a master regulator of the SMC phenotype.

In an effort to define the cells of the cardiovascular system derived from Myocd-dependent lineages, we performed lineage tracing in mouse embryos by introducing Cre recombinase into

the Myocd locus and monitoring the expression of a Cre-dependent lacZ from the ROSA26 reporter (R26R) mouse line. Consistent with previous expression data, cardiac and vascular SMCs are derived from Myocd-dependent lineages. Surprisingly, skeletal muscle in these mice also expressed lacZ, indicating its derivation from a Myocd-dependent lineage. However, rather than functioning as an activator of skeletal muscle gene expression, Myocd represses MyoD-mediated stimulation of the Myog promoter and blocks skeletal muscle differentiation *in vitro*. At the same time, Myocd transactivates SMC contractile protein genes, thereby converting skeletal myoblasts to an SMC phenotype. These results suggest that Myocd acts as a bifunctional switch for muscle differentiation by concurrently opposing the gene program for skeletal muscle differentiation and specifying a SMC fate.

Results

Myocd Is Expressed in Progenitors of Skeletal Muscle. Myocd is expressed throughout the atrial and ventricular myocardium and in a subset of vascular and visceral SMCs (8). To trace the embryonic origins of Myocd-expressing lineages, we performed lineage tracing by creating a mouse in which the first exon of Myocd was replaced with a Cre-recombinase cassette [supporting information (SI) Fig. 7]. Hemizygous Myocd-Cre knockin mice were crossed into the R26R mouse line, allowing for persistent activation of lacZ expression after Cre-mediated excision. As expected from prior *in situ* hybridization studies of Myocd expression (8), we observed lacZ staining in the developing heart, dorsal aorta, and head mesenchyme during early development (Fig. 1). LacZ expression was first seen in cardiomyocytes of the cardiac crescent at embryonic day 7.5 (E7.5) and subsequently in the linear heart tube and in all chambers of the heart (Figs. 1 and 2). We also noted expression of lacZ in SMCs of the embryonic and adult aorta and other vascular structures such as renal arterioles (Fig. 2).

Unexpectedly, lacZ-positive cells were observed in the somites of mouse embryos beginning at E8.5 and throughout mature skeletal muscle fibers at later stages (Figs. 1 and 2). Histological sections of an E9.0 embryo further confirmed lacZ-stained skeletal progenitors within the dermamyotome of the somites, the origin of epaxial and hypaxial skeletal muscle (Fig. 2 C and D). Expression also was observed in a subset of sclerotome cells.

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Abbreviations: MRF, myogenic regulatory factor; MEF2, myocyte enhancer factor-2; SRF, serum response factor; SMC, smooth muscle cell; Myog, myogenin; Myocd, myocardin; Myh11, smooth muscle myosin heavy chain; TAD, transactivation domain; HDAC, histone deacetylase; En, embryonic day *n*.

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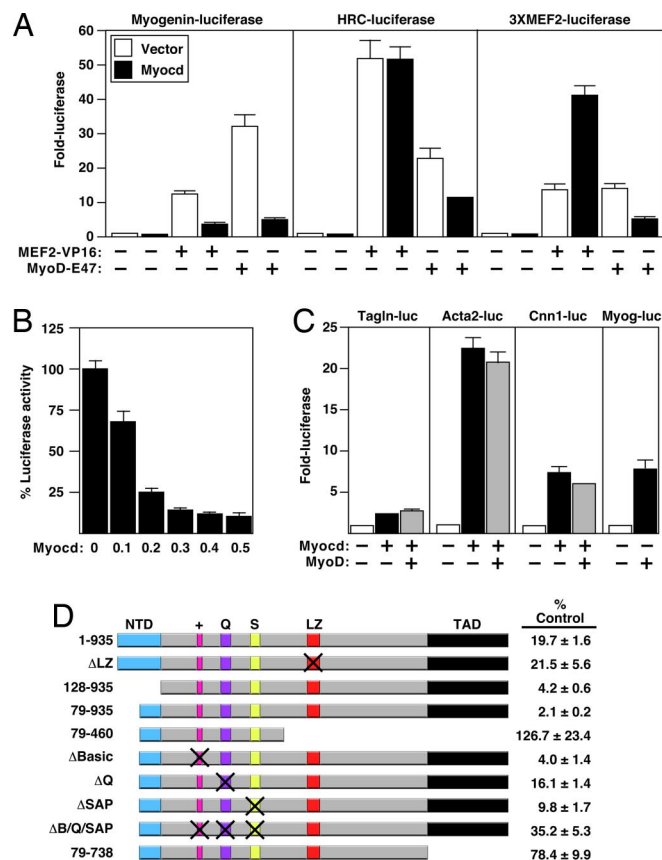


Fig. 4. Myocd represses the Myog promoter. (A) C₂C₁₂ myoblasts were cotransfected with the indicated luciferase reporters and myogenic activators in the absence (open bars) or presence (filled bars) of Myocd. Luciferase activity is expressed as normalized fold increase above baseline (no myogenic activators) set to 1. (B) C₂C₁₂ myoblasts cotransfected with -660 Myog promoter-luciferase (Myog-Luc) and MyoD-E47+MEF2C in absence or presence of increasing concentrations of Myocd. Luciferase activity is expressed as a percentage of control set to 100. (C) PAC1 cells cotransfected with the indicated SMC promoters linked to luciferase in absence or presence of Myocd ± MyoD. Luciferase activity is expressed as normalized fold increase to each luciferase reporter alone (set to 1). The Myog promoter is shown as a positive control for MyoD activation. (D) C₂C₁₂ myoblasts cotransfected with Myog-Luc and MyoD-E47+MEF2C in the presence of pcDNA control vector or each indicated Myocd expression plasmid (expression validated by Western blotting) (data not shown). The domains of the Myocd expression plasmids are indicated with colored boxes labeled as N-terminal domain (NTD), basic (+), poly glutamine (Q), SAP (S), leucine zipper (LZ), and TAD. The numbers at right reflect the percentage normalized luciferase activity for each Myocd construct relative to the pcDNA control vector alone (set to 100). All data are representative of two independent experiments.

increase in Myh11 expression (SI Fig. 8C). These results were confirmed by immunostaining (Fig. 3C). All stable clones expressing Myocd were unable to differentiate into myotubes (Fig. 3Cf and SI Fig. 8D).

We considered the possibility that diminishing myocardin levels in cultured SMCs might render them susceptible to transdifferentiation to a more skeletal muscle fate through up-regulation of Myog. Indeed, overexpression of Myocd in PAC1 cells, which display features of both smooth and skeletal muscle (14), stimulated Myh11 and attenuated Myog expression (Fig. 3D), whereas siRNA knockdown of Myocd augmented expression of Myog mRNA (Fig. 3E). We conclude that Myocd is a repressor of skeletal muscle differentiation *in vitro*.

Myocd Represses MyoD-Dependent Activation of the Myog Promoter. To determine whether Myocd repressed Myog expression at the transcriptional level, we evaluated effects of Myocd on activation

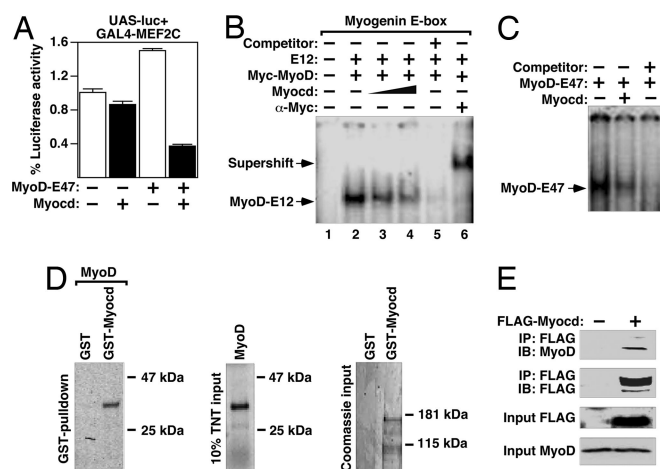


Fig. 5. Myocd interferes with MEF2-MyoD association and MyoD DNA binding. (A) 10T1/2 cells were cotransfected with UAS-Luciferase reporter and GAL4-MEF2C ± MyoD-E47 in the absence (open bars) or presence (filled bars) of Myocd. Luciferase activity is expressed as a percentage of the control (first bar set to 1). (B) Gel-shift assay with radiolabeled E1-box of Myog promoter incubated with *in vitro* translated Myc-MyoD, MyoD's dimerization partner, E12 ± Myocd. The MyoD-E12 nucleoprotein complex is reduced in the presence of *in vitro*-translated Myocd and is supershifted with an Myc antibody. (C) Gel-shift assay by using an oligonucleotide corresponding to the E-box in the MCK promoter incubated with nuclear extracts from 10T1/2 cells cotransfected with the tethered MyoD-E47 expression plasmid ± Myocd. Note attenuated MyoD-E47 complex in the presence of Myocd. (D) (Left) GST pull-down assay shows Myocd interacting with *in vitro* translated MyoD. (Center and Right) Validation of the presence of each protein by autoradiography and Coomassie blue staining, respectively. (E) C₂C₁₂ myoblasts transfected with FLAG-tagged Myocd and then immunoprecipitated with a Flag antibody, followed by immunoblotting as indicated. Results are representative of two independent studies.

of the Myog promoter, which contains MyoD-binding E-box elements and an MEF2 site (15). Both MEF2- and MyoD-dependent activation of the Myog promoter were reduced in the presence of Myocd (Fig. 4A Left), and such repression was dose-dependent (Fig. 4B). Myocd could repress the Myog promoter irrespective of point mutations in each myogenic factor regulatory element, as well as a conserved upstream CARG box (SI Fig. 9). Myocd had no effect on MEF2-dependent stimulation of the histidine-rich calcium-binding protein promoter (Fig. 4A Center) (16) and activated a synthetic MEF2-dependent reporter (Fig. 4A Right), suggesting the block in Myog promoter activation is not a consequence of generalized transcriptional squelching or an inhibition in MEF2 DNA binding. In contrast to MEF2, when a tethered MyoD-E47 dimer (17) was used as the myogenic activator, Myocd repressed all three promoters (Fig. 4A). Remarkably, MyoD had a minimal effect on Myocd's ability to activate several SMC-specific promoters (Fig. 4C). Collectively, these results indicate that Myocd transcriptionally represses MyoD-mediated Myog promoter activity in a CARG/SRF-independent manner, whereas MyoD is ineffective in blocking Myocd transactivation of SMC promoters.

Multiple Domains in Myocd Are Necessary for Repression of the Myog Promoter. Alternate and mutant forms of Myocd were tested for transcriptional repression of the Myog promoter. The long form of Myocd (amino acids 1–935), shown previously to interact with MEF2 (11), inhibited Myog promoter activation by 80% (Fig. 4D). Two shorter forms of Myocd (amino acids 128–935 and 79–935) were 5–10 times more potent in repression, suggesting

amounts of p300. Because the TAD of Myocd is essential for its transrepression activity, this domain may interact with another protein to mediate transcriptional repression. One potential candidate is MyoD, which we show by GST pulldown and coimmunoprecipitation assays to physically associate with Myocd. MyoD–Myocd complexes likely account for attenuated MEF2–MyoD functional association and reduced MyoD binding to E-boxes in the *Myog* promoter.

Skeletal muscle differentiation and the MRFs are considered to be dominant over other cell types, including cardiac and smooth muscle (2, 23). Importantly, the apparent dominance of the skeletal muscle program over SMCs only has been shown *in vitro*, where levels of Myocd are low. We suggest that the stoichiometry of Myocd is critical in maintaining SMC differentiation. When Myocd levels are low, SMCs lose their differentiated phenotype and may take on other cell fates. However, when Myocd levels are elevated, cells are more likely to adopt an SMC fate. Interestingly, we have shown MyoD to have no effect on Myocd-dependent transactivation of SMC-restricted promoters, suggesting that Myocd can override the actions of MRFs and the skeletal muscle program of differentiation.

Myocd as an Early Marker of the Skeletal Muscle Lineage. Retrospective clonal analysis in mice has shown some embryonic aortic SMCs to arise from the dermamyotome of somites, thereby providing evidence for the existence of a common progenitor for smooth and skeletal muscle (24). Recently, quail-chick transplantation studies also found the sclerotome compartment of the somite to be a source of aortic SMCs (25). We detected lacZ expression directed by Myocd-Cre in both the dermamyotome and the sclerotome. Regardless of the somitic origins of aortic SMCs, the expression of Myocd-Cre suggests that Myocd is expressed in somitic progenitor cells that may give rise to aortic SMCs. Therefore, it is possible that a subpopulation of Myocd-expressing cells within the somites is prevented from differentiating into myotubes or other cell types (e.g., bone), thus allowing these cells to migrate to the dorsal aorta and differentiate into vascular smooth muscle. Because Myocd expression is not detected in the skeletal muscle lineage beyond E9.0 (8), we propose that Myocd is required only transiently in a common progenitor of skeletal and smooth muscle lineages, and that its subsequent repression is required for skeletal muscle development.

There are several instances during development where SMCs transdifferentiate into skeletal muscle (26–28). We hypothesize that a critical prerequisite for SMC–skeletal muscle transdifferentiation is the silencing of Myocd expression. In support of this premise, cultured SMCs with a propensity to transdifferentiate into skeletal muscle (14) display low-level expression of Myocd

(20). It will be interesting to investigate whether the types of repressive mechanisms observed here also are operative in settings of SMC phenotypic modulation, as occurs during pathological vascular remodeling *in vivo*. Finally, VEGF can promote the transdifferentiation of skeletal myoblasts or muscle-derived stem cells into functional SMCs (29). Because VEGF is under study in a variety of angiogenesis clinical trials, it may be prudent to evaluate skeletal muscle function in patients undergoing this type of therapy.

In summary, we identified a new function of Myocd related to the transcriptional repression of Myog and the respecification of skeletal myoblasts to a SMC-like lineage. We propose that Myocd functions as a bifunctional molecular switch for muscle differentiation, advancing SMC differentiation while repressing the skeletal muscle differentiation program. These studies have important implications for understanding the molecular underpinnings associated with transdifferentiation of skeletal muscle and smooth muscle during development and the derivation of these cell types from stem cells.

Materials and Methods

PCR primers and antibodies are listed in [SI Tables 1 and 2](#).

Generation of Myocd-Cre Knockin Mice. The Myocd-Cre knockin mouse was created with standard methods detailed in [SI Materials and Methods](#).

Cell Culture. Cells were grown without antibiotics at 37°C in 10% FBS. C₂C₁₂ myoblasts were induced to differentiate with 2.5% horse serum for 72 h. Transfections, transductions, and derivation of stable cell lines were done with standard procedures described in [SI Materials and Methods](#).

Expression Analyses. RT-PCR, Western blotting, and immunofluorescence microscopy were done on various cell types with standard methods as detailed in [SI Materials and Methods](#).

DNA–Protein and Protein–Protein Interaction Assays. Gel-shift, GST pulldown, and coimmunoprecipitation assays were done with established techniques as detailed in [SI Materials and Methods](#).

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